IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 CFR 1.131

The undersigned declare as follows:

1. We are co-inventors of the above-identified patent application assigned to The Johns Hopkins University.

2. Prior to September 1998, we conceived of and then diligently reduced to practice the methods of introducing into endothelial cells of an autologous vein grafts from a mammal an effective amount of at least one nucleic acid encoding thrombomodulin (TM), NF-κB inhibitor, or a functional fragment of TM; provided that when the agent is thrombomodulin, the nucleic acid further encodes the NF-κB inhibitor, wherein the introducing is performed ex vivo or by direct injection into the graft, and transplanting the vein graft into the mammal as disclosed and claimed in the above-identified patent application.

3. We diligently worked to reduce the methods to practice until the filing of the provisional application on May 22, 2000.

5. Attached as Exhibit 1, Figures 1 – 20, are true and accurate copies of laboratory notebook records with dates deleted. The notebook records demonstrate the conception, reduction to practice and diligence from conception to the filing of the application. The exhibits show that the constructs for the expression were received prior to September 1998 representing proof of the conception of the methods of using thrombomodulin to prevent early vein graft thrombosis as described in paragraph 2 above (Figure 1).

6. Figure 2 shows successful adenovirus-mediated gene transfer of and expression of B-galactosidase marker genes in rabbit vein grafts. Figure 3 demonstrates that we were also transducing rabbit vein graft with the adenovirus vector expressing human TM (AdTMh5; Figure 3). Figure 4 demonstrates that we were able to quantify native TM expression in vein grafts by harvesting rabbit vein grafts at various time points. Figure 5 is a page from Dr. Antony Kim's notebook demonstrating TM expression over time (TMTC series of rabbits) outlining a visual scoring system devised to quantify native TM expression in rabbit vein grafts.

7. Figure 6, a page from Dr. Rade's notebook, depicts the continued generation of rabbits for TM quantification (C6W) and the transduction of rabbit vein grafts with control adenovirus (Adl312). Figure 7, a page from Dr. Kim's notebook, details a digital scoring system for TM expression in vein grafts. Figure 8, a log from Dr. Rade's notebook demonstrates transduction of a series of rabbit vein grafts with a different control adenovirus (AdRNull-1). Figures 9 and 10, from Dr. Kim's notebook, document TM expression in rabbit vein grafts using the digital imaging system.

8. Figure 11, a page from Dr. Rade's notebook, shows a series of rabbit vein grafts transduced with AdTMh5. Figures 12-14, from Dr. Kim's notebook, document the measurement of protein C activation and human TM protein expression in rabbit vein grafts transduced with either the AdTmH5 or control adenoviral vectors. Figure 15, a page from Dr. Richard Sohn's notebook, demonstrates the construction of an adenoviral vector expressing IkB, a potent NF-κB inhibitor. Figures 16, a page from Dr. Richard Sohn's notebook, shows the measurement of NF-κB activation in rabbit vein grafts.

9. Figure 17 documents the ability of the IκB adenovirus in preventing TM down-regulation in response to inflammatory cytokines. Figure 18, a page from Dr. Rade's notebook, details the group of rabbit vein grafts that were transduced with the IκB adenovirus. Figure 20 is

a figure resulting from data demonstrating that over-expression of IκB can reduce neointimal formation in rabbit vein grafts. Figure 21, a page from Dr. Sohn's notebook, shows that over-expression of IκB also effectively inhibits NF-κB activation in rabbit vein grafts.

10. We hereby further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both (18 U.S.C. 1001), and that such willful false statements may jeopardize the validity of the above-identified application or any patent issued thereon.

Date: 10/30/06 Jeffrey J. Rade

Date: _____ Antony Kim

Date: 10/30/06 Richard Sohn

a figure resulting from data demonstrating that over-expression of IκB can reduce neointimal formation in rabbit vein grafts. Figure 21, a page from Dr. Sohn's notebook, shows that over-expression of IκB also effectively inhibits NF-κB activation in rabbit vein grafts.

10. We hereby further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both (18 U.S.C. 1001), and that such willful false statements may jeopardize the validity of the above-identified application or any patent issued thereon.

Date: _____ _____
Jeffrey J. Rade

Date: 2 NOV 2006 _____
Antony Kim

Date: _____ _____
Richard Sohn

Figure 1

Project No. 2
Book No. 1 TITLE Cloning of human Thrombomodulin into AdLOX

From Page No.

Purpose: To make recombinant Adenovirus c human thrombomodulin

1. Obtained plasmid clone of human Tm from ATCC (ATCC#61348)

ATCC 61348, 61349 - plasmid clone of human thrombomoduli... http://www.atcc.org/cgi-bin/SFgate?lang...828%20%2fpub%2ftextfiles%2fMB-CLONE.TXT

Your query was:
thrombomodulin

Conf. # 5044066
$105 + $17.75 shipping

ATCC 61348, 61349 - plasmid clone of human thrombomoduli

ATCC 61348, 61349 - plasmid clone of human thrombomodulin, THBD
NAME: puc19TM15 (GDB:168893) [lambda HTm15]
DATABASE ACCESSION: DNA Seq. Acc. M16552
VECTOR: plasmid vector: pUC19
ORGANISM: THBD: Homo sapiens (human)
TISSUE: umbilical vein endothelial cells
GENE NAME: in insert THBD: thrombomodulin (GDB:119613)
CHROMOSOME: THBD 20-p11.2
DNA: THBD cDNA
CONSTRUCTION: Insert lengths (kb): THBD 3.70
Excise by: EcoRI or SalI
6.40
Sequence Position: DNA Seq. Acc.: M16552:
MARKERS: ampR
DEPOSITORS: J.E. Sadler
REFERENCES: Biochemistry 26: 4350-4357, 1987
J. Biol. Chem. 264: 20705-20713, 1989 (CIT:381458)
Genomics 5: 649-650, 1989 (CIT:13744)
DESCRIPTION: Restriction digests of the clone give the following sizes (kb): EcoRI--3.7, 2.7; SacI--6.5, AvaI--3.4, 2.0, 0.2; XbaI--5.9, 0.54, 0.2, 0.1. (ATCC staff)
There is 64% homology between this probe and bovine thrombomodulin. A single band of 3.7 kb is detected in human placenta and endothelial cell poly(A)+ RNA. The insert includes 146 nt of 5'-noncoding sequence, an open reading frame of 1725 nt and 1779 nt of 3'-noncoding sequence including 40 nt of poly(A) tail. The insert contains the following sites separated by (bp) (approx): EcoRI- 270- SmaI- 160- PstI- 240- PstI- 210- PstI- 100- PstI- 530- KpnI- 310- PstI- 1080- HindIII- 110- BamHI- 205- PstI- 475- EcoRI. (Biochemistry 26: 4350-4357, 1987)
GROWTH CONDITIONS: Medium 1227 37C
SHIPPED: 61348: freeze-dried Escherichia coli SURE
61349: dried purified DNA (200 ng)
PRICE CODE: 61348 D - TIGR/ATCC SPECIAL COLLECTION OF HUMAN cDNA CLONES
; 61349 D - TIGR/ATCG SPECIAL COLLECTION OF HUMAN cDNA CLONES

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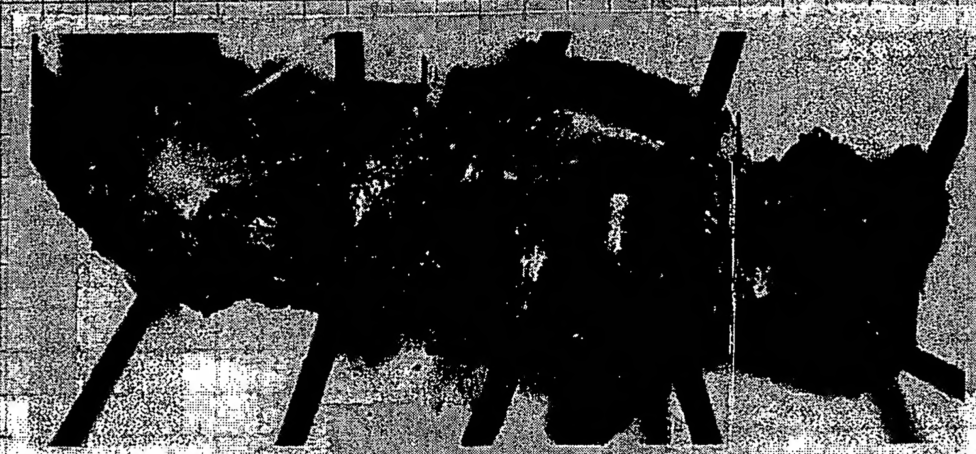
Figure 2

From Page No. Ø

Purpose: Stain vein graft segments c̄ X-Gal, fix c̄ 2% Formaldehyde / 0.2% Glutaraldehyde, open longitudinally & photograph c̄ digital camera.

① BGAV-1
Implant [redacted] → Harvest [redacted]
Tx c̄ 200 µl M-199 + Ad CMVLacZ (50 µl [redacted] 8x10^10 PFU/...
→ 4x10^9 PFU (2x10^10 PFU/ml)



Mid-Tox...

Blue suture = prox anastomosis arrows.

② BGAV-4
[redacted] → [redacted] same way

Mid-Tox...

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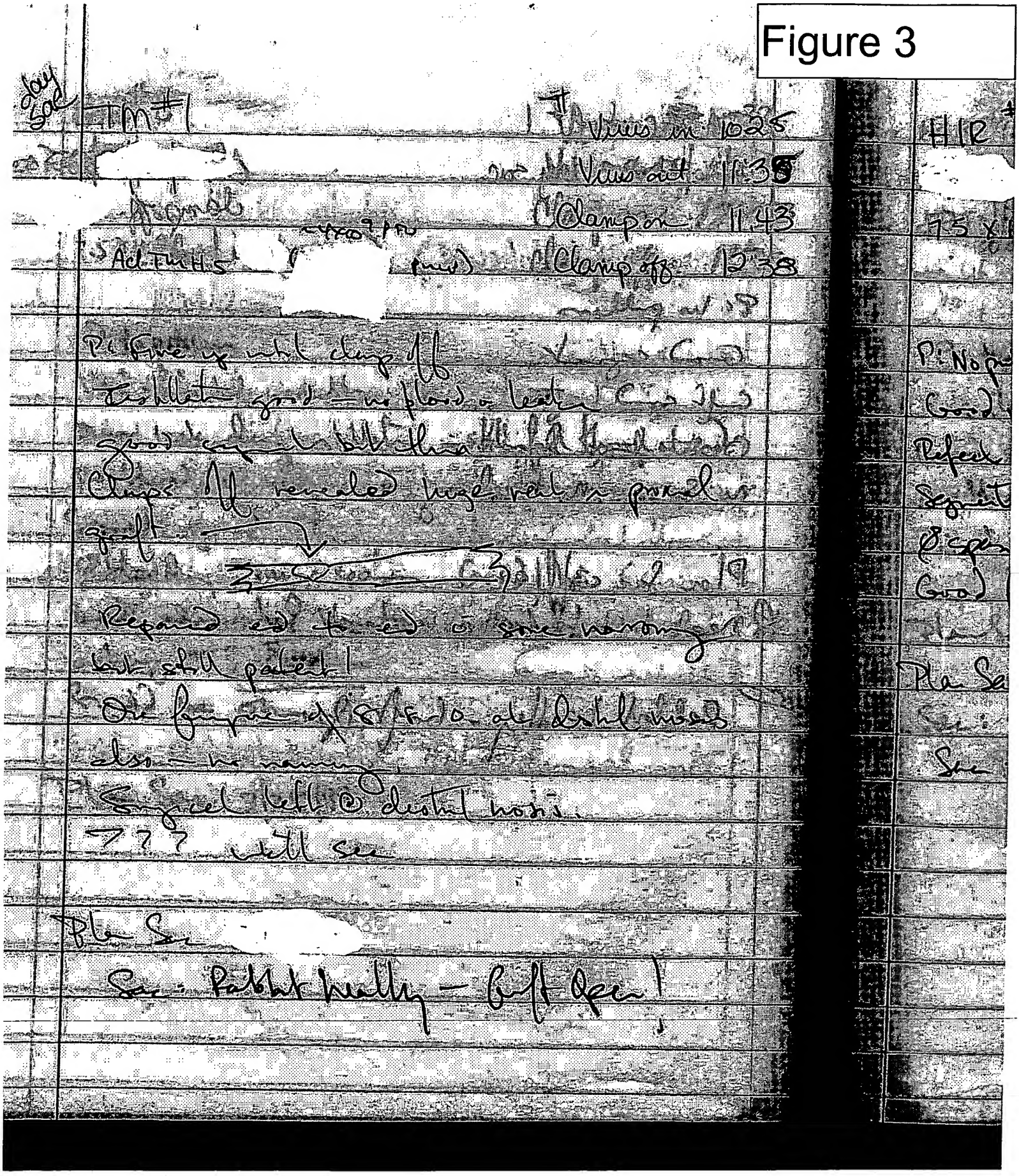
Figure 3


day 5
TM#1
Vein in 10:25
Vein out 11:38
Clamp on 11:43
Clamp off 12:38
HR
75 x
P: No
Good
Repeat
Squint
Good
Pla Sa
Sac

Project No.

Book No. TITLE

From Page No. 7?

GRAFTS SENT TO ARI

Specimen	Surgery Date	Harvest Date	Days	Dose	A…
TMTC-1			5	φ	25
TMTC-2			5	φ	25
TMTC-3			5	φ	25
TMTC-4			7	φ	25
TMTC-5			7	φ	25
TMTC-6			7	φ	25
TMTC-7			1	φ	258
TMTC-8			1	φ	258
TMTC-9			1	φ	258
TMTC-10			3	φ	258
TMTC-11			3	φ	25

SENT

TMTC-12A			1D	φ	SEM
TMTC-12B			1D	φ	SEM
~~TMTC-13~~			28d		
~~TMTC-14~~					
TMTC-15			3	SEM	
TMTC-16			3	SEM	

SENT

TMTC-13			28d	φ	SEM
TMTC-17			14d		SEM
TMTC-18			5d	SEM	
TMTC-19			1d		
~~TMTC-20~~					

AAULAC2-1			28d		AAULAC2
AAULAC2-2			14d		"
AAULAC2-3			7d		"

→ SENT

TMTC-21

TMTC-23

14d

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Figure 5

TITL Rabbit Vein Graft Analysis (cont'd) Project No.

Book No.

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Grading System (cont'd)

classification of thrombomodulin expression (intensity)

class	description
1+	trace, minimal
2+	patchy, scattered
3+	confluent, but not maximal intensity
4+	maximum intensity

Raw Data & Mean + S
(compiled & Peter

CIRC.	day1		INTENS.	day1	
	2	2		2	2
	3	3		2	2
	3	3	02/03		2.5
avg		2.666667	avg		2.166667
STD		0.57735	STD		0.235702

	day3			day3	
	2	2	02/01		1.5
	2	2		1	1
avg		2	avg		1.25
STD		0	STD		0.25

	day5			day5	
	3	3		2	2
	2	2		2	2
02/03		2.5		2	2
avg		2.5	avg		2
STD		0.408248	STD		0

	day7			day7	
02/03		2.5		2	2
	3	3	02/03		2.5
	3	3		2	2
avg		2.833333	avg		2.166667
STD		0.235702	STD		0.235702

	day28				
	4	4			
	3	3			
	3	3			02/0
	3	3			
03/04		3.5			
	3	3			
avg		3.25			avg
STD		0.381881			STD

	control			
	4	4		4
	4	4		03/04
	4	4		3
avg		4		avg
STD		0		STD

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Book No. TITLE

Figure 6

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GUARDS SENT TO APIB

Specimen	Source Date	Harvest Date	Days	Dose	Virus
2855 C-32, LT-13			28d	φ	φ
2856 C-33, LT-14			28d	φ	φ
2857 C-34, LT-15			28d	φ	φ
Adcon-1 2858			28d	7.5x10⁹	Adl
Adcon-2 2859					
Adcon-3 2860					
Adcon-4 2861					
Adcon-5 2862					
Adcon-6 - not sent					
Adcon-7 2863					
TMTC-22 2864			14d	φ	φ
TMTC-24 2865			3d	φ	φ
=					
TMTC-21 3062			14d	φ	φ
TMTC-23 3061			14d	φ	φ

Frank Hand Carried

Specimen	Source Date	Harvest Date	Days	Dose	Virus
=					
Adcon-8 3205			28d	7.5x10⁹	Ad lacZ
Adcon-9 3206			"	"	
TMTC-25 3207			14d	φ	φ
C6W-2 3208			6wk	φ	φ
C6W-3 3209			6wk		
C6W-5 3210			6wk		
C6W-8 3211			6wk		
C6W-10 3212			6wk		

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Figure 7

104 Project No._____ Book No._____ TITLE TM in RVG model

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Digital Imaging of Thrombomodulin in RVG's:

Protocol: Hypothesis #1: TM protein expression decreases in RVG over time when grafted into arterial circulation.

Reason:

Hypothesis #2: TM protein production shifts the thrombo-resistant balance in milieu of normal vasculature to cause a more favorable thrombotic state.

Reason:

Hypothesis #3: The prothrombotic state left by the reduction of TM is a factor in VG failure

Reason:

Adobe
1. shoot images via DMC
2. adjust brightness in order to control for all vessels
3. Mask the vessel ~~~ from endothelium

Sigma Scan
4. measurement setting → # of pixels (area) calibration 333 to 1
5. measure # of pixels on vessel endothelium (choose range for signal intensity threshold)
6. measure circumference of endothelium

Quattro
7. Record values including # pixels, circumference, + P/C

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Figure 8

GRAFTS SENT TO API

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Specimen	Survey	Hours	Days	Virus	Dose	
Adcon-10			28	Adenovirus-1	3.5×10^9	345
Adcon-11						345
Adcon-12						346
Adcon-13						347 Simian
Adcon-14						345
Adcon-15						345
Adcon-16						345
Adcon-17						345
Cbuw-13			6 wks	ϕ	ϕ	3459
Cbuw-14						3456
Cbuw-15						3456
Cbuw-16						3456
Cbuw-17						3456

SENT

FROM SECTIONS; GRAFT, ARTERY, CONTROL ARTERY

C-35	1d	ϕ	ϕ
C-36	1°		
C-37 C-38 Cbuw-18	6 wks	1	1

FROM HMO CANCER TO API

FROM SECTIONS; GRAFT, ARTERY, CONTROL ARTERY, VEIN

C-39	7d	ϕ	ϕ
C-40	7d	ϕ	ϕ
C-41	3d	GRAFTS CLOTTED	
C-42	1d	CONTROL ART, VEIN ONLY	
C-43	1d	GRAFT, ART, VEIN	
C-44	7d		
C-45	7d		
	1°	GRAFT ONLY	

1° 1d 3d 7d 14 28 64

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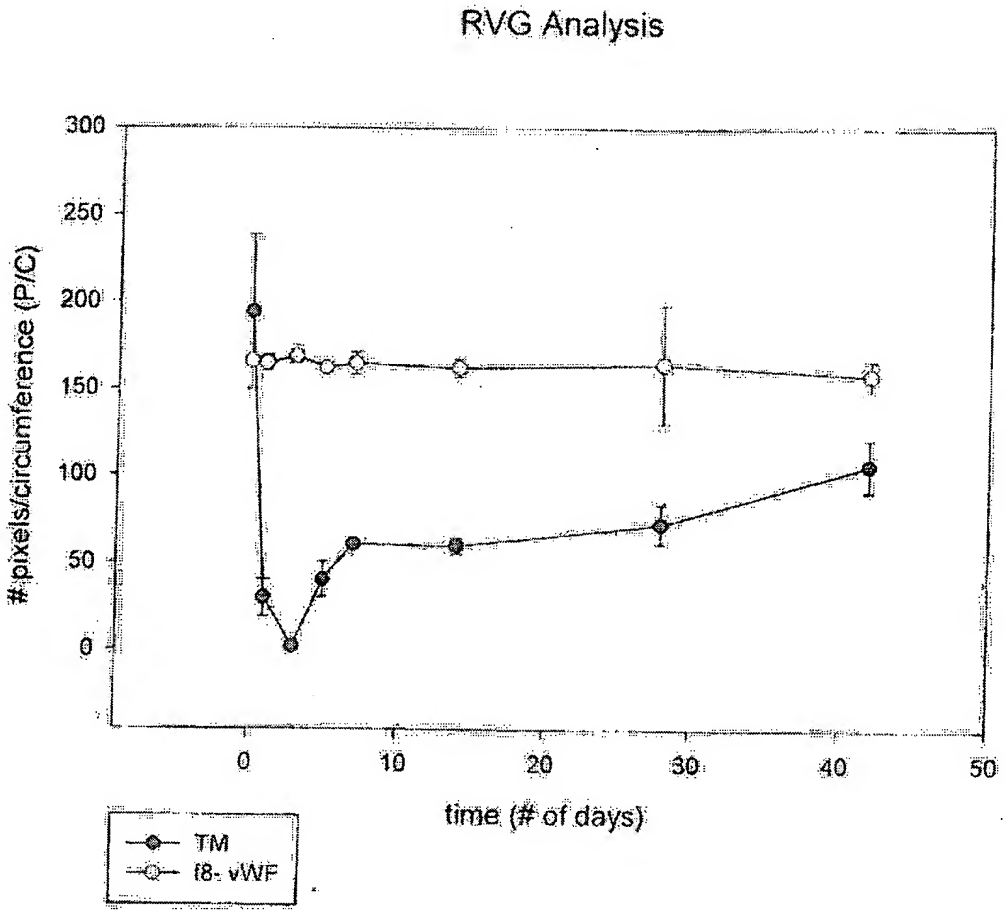
Figure 9



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Book No.

Figure 10

Page No.

TM & Factor 8 strains

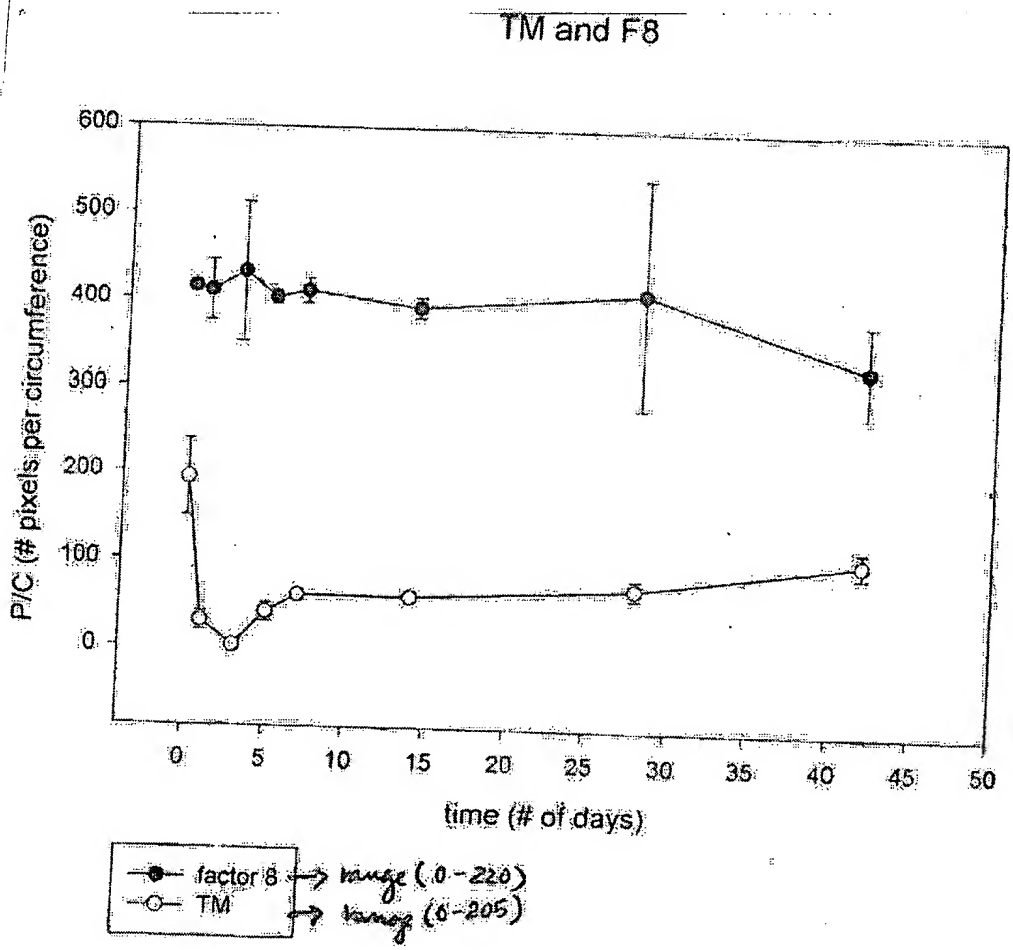

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Figure 11

100 Project No.________ Book No.________ TITLE Grafts in Swt: to APID

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		Strain	Site	Virus	Dose
	Perfusion Fixing 10% Formalin → 70% EtOH For morphology				
4017	AdTmHS #3				
4018	AdTmHS #4			AdTmHS	3.75 x10^10 pt/ul
4019	AdTmHS #5			|	
4020	AdTmHS #6			|	
4021	AdTmHS #8			|	

Swt

=

Perfusion Fixing 10% Form → 70% EtOH

Perfusion Fixing 10% Formalin → 70% EtOH For morphology

		Virus	Dose
4232	AdTmHS #12	AdTmHS	3.75x10^10 pt/ul
4233	AdTmHS #15	|	
4234	AdTmHS #16	|	

Hand carried to APID

=

		Virus	Dose
4508	C-89		
4509	C-90		
4510	Adcon-33	AdRelux-1	3.75x10^10 pt/ul
4511 / 4600	Adcon-36	|	|

Perf Fixed 10% Form → 70% EtOH

Swt

=

		Virus	Dose
4430	AdTmHS #14	AdTmHS	3.75x10^10 pt/ul
4431	Ad con #21	AdRelux-1	|
4432	C-74	—	—
4433	C-81	—	—

Perfusion Fixing 10% Form → 70% EtOH

Swt

=

		Virus	Dose
4577	Adcon 35	AdRelux-1	3.75x10^10 pt/ul
4578	Adcon 42	"	"
4579	C-91	—	—
4580	C-92	—	—
4581	C-93	—	—

10% Form Fixed 70% EtOH Swt

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Figure 12

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Book No._____ TITLE PC Assay + Fbn Act. Assay

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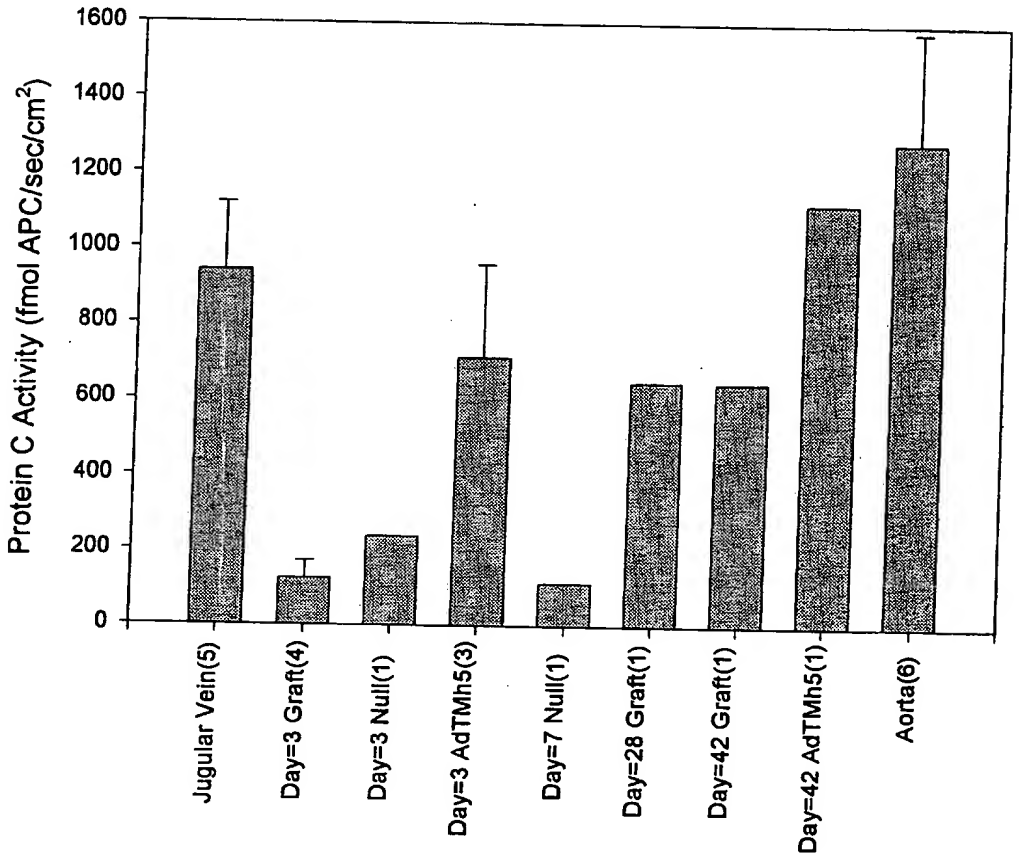
Thurs. Protein C Assay (Graph)



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Q PC Activation Assay :

Figure 13

PC Activation Assay AdTMh5 #25 (day 7)

[thrombin] nM	10
[PC] uM	1

	RVG (7 day)	blank
AdTMh5#25	522	16.29
AdTMh5#25 - blank	505.71	
avg.	505.71	16.29
aPC	0.468275065	
aPC(fmoles/min/cm2)	2498.2474717	

Q

aPC Standard Curve

uM aPC	mOD/min
0	0.857
0.05	55.4
0.1	107.4
0.2	213.8
0.3	311.6
0.4	428.4
0.5	549.2

Regression Output:

Constant	0.001813143
Std Err of Y Est	0.006520627
R Squared	0.998984198
No. of Observations	7
Degrees of Freedom	5
X Coefficient(s)	0.00092239
Std Err of Coef.	1.31539E-05

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Mon TM ELISA for AdTMh5 + AdNull (American diagnostica inc kit #837

Figure 14

THROMBOMODULIN ELISA

	sample	OD (A450)	Dilution factor	TM	corrected TM	TM/BSA
AdTMh5						
	day 3					
	TM9	1.21	20	3.20804	24.20000	39.18483
	TM11	1.23	50	3.27142	163.57088	185.85785
	TM23	1.359	50	3.68020	184.00989	108.11640
	day 7					
	TM25	3.166	50	9.40629	470.31439	354.42972
	TM27	2.466	50	7.18810	359.40506	149.04811
	day 14					
	TM19	0.681	10	1.53173	15.31726	9.36070
	TM20	0.781	10	1.84861	18.48609	7.40715
	TM21	0.791	10	1.88030	18.80298	9.96799
	day 28					
	TM22	2.187	50	6.30400	315.19977	231.53710
	TM29	1.162	50	3.05594	152.79683	66.64825
	TM30	1.148	50	3.01157	150.57865	65.59129
	day 42					
	TM18	0.437	10	0.75853	7.58529	11.80432
AdNull						
	day 3					
	NULL23	0.187	2	0	0	0
	NULL28	0.16	2	0	0	0
	NULL43	0.204	2	0	0	0
	day 7					
	NULL25	0.195	2	0	0	0
	NULL27	0.206	2	0	0	0
	NULL31	0.201	2	0	0	0
	day 14					
	NULL34	0.258	2	0	0	0
	NULL37	0.193	2	0	0	0
	NULL40	0.149	2	0	0	0
	day 28					
	NULL29	0.172	2	0	0	0
	NULL36	0.194	2	0	0	0
	NULL39	0.165	2	0	0	0
	day 42					
	NULL19	0.181	2	0	0	0
	NULL22	0.197	2	0	0	0
	NULL24	0.188	2	0	0	0

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Figure 15

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Primer "RSV1": AGCACCGTGCATGCCGATTGGTGGA

To insert present, then fragment → ~ 1300 - 1400 bp.

PCR: ① RSV WT ② RSV ΔS ③ pAdlox R. IKB wt

Viral plates: overlayed 2nd layer MEM/agarose.

- No insert in viruses
- ⊖ Ctrl (shuttle plasmid) verifies PCR works

→ Passed out plates ✓ for CMV, ΔS IKB

① Picked 6 plaques and inoculated 6 well plate ∴ 293 cells.

② Thawed CTC ∴ cells

③ Digested [pAdlox R IKB wt / pAdlox R IKB ΔS] on hope that recombination ∴ YS would be more efficient.

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Figure 16

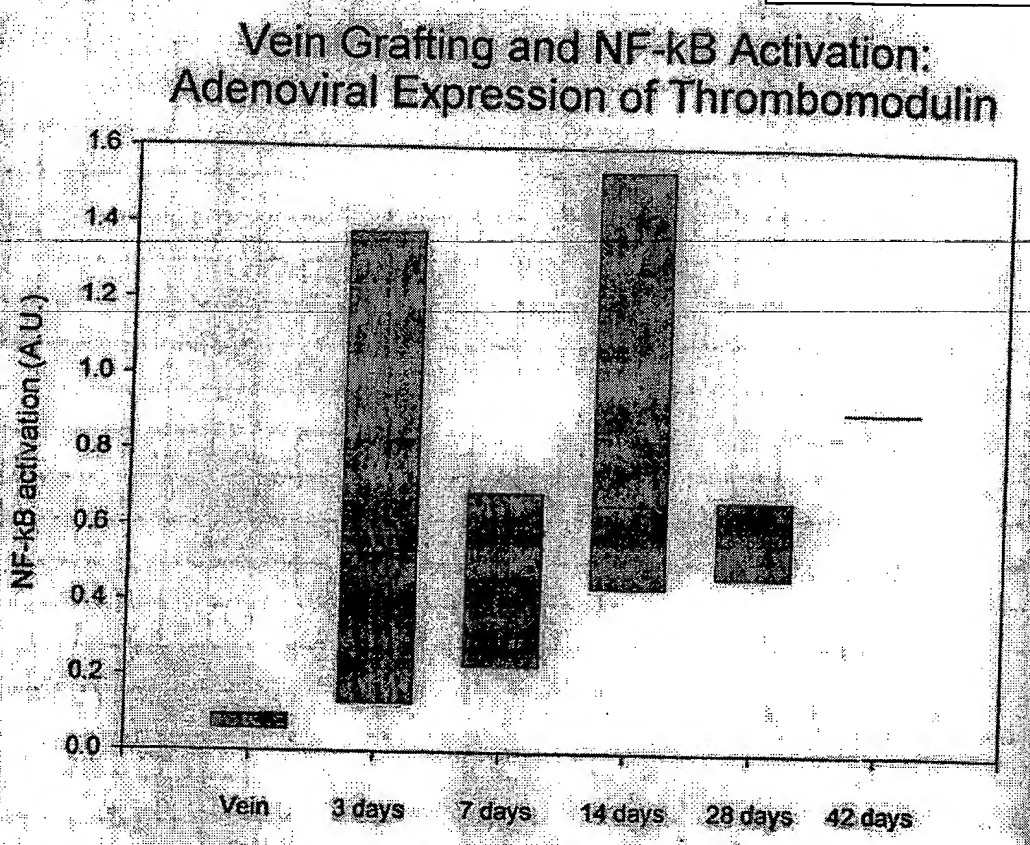

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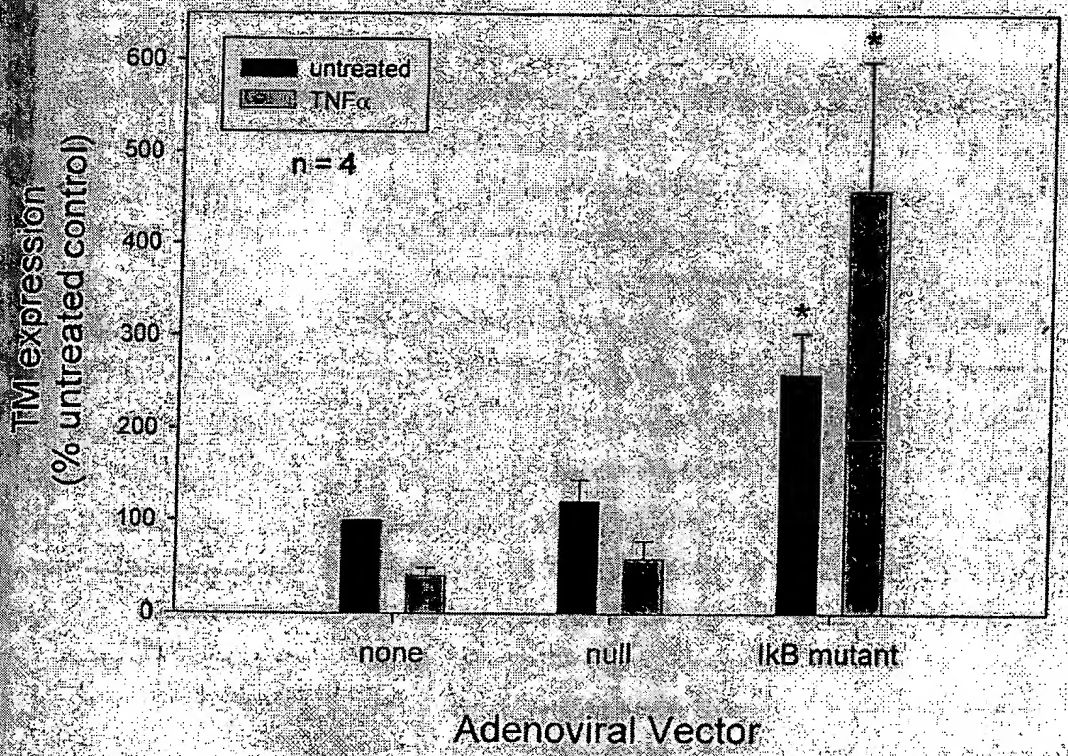
Figure 17

	ctrl	TNF	null	null + TNF	IkBm	IkB + TNF
gel 1	598	274	519	148	1262	1290
gel 2	249	63	430	228	831	1826
gel 3	286	84	423	265	950	1918
gel 4	1088	668	841	288	1754	2330
as % ctrl	100	45.8194	86.7893	24.74916	211.0368	215.7191
	100	25.3012	172.6908	91.56627	333.7349	733.3333
	100	29.37063	147.9021	92.65734	332.1678	670.6294
	100	61.39706	77.29779	26.47059	161.2132	214.1544
mean	100	40.47207	121.17	58.86084	259.5382	458.459
S.D.	0	16.53125	46.46073	38.40392	87.17875	282.3589
S.E.M.	0	8.265623	23.23036	19.20196	43.58938	141.1794
t-test						
vs. control			0.397268		0.010579	
vs. null					0.031108	
vs. null-TNF						0.030975

Inhibiting NF-kB Activation In HUVEC Upregulates TM and Prevents TM Downregulation By TNFα



* $P < 0.05$, compared to respective null-transduced

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[redacted]

Figure 18

Project No.______

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Book No.______ TITLE GRAPHS SENT TO APP

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10% Form Fixed → 70% EtOH

RABBIT		Study	Handler	Virus	Dose	Time
11CB-2	6274			AdRyKB-1(32/36)	3.75x10^9 PAU/kg	6wks
11CB-7	6275					
11CB-9	6276					
11CB-10	6277					
11CB-11	6278					
11CB-12	6279					
11CB-13	6280					

Fendeker

Study Ha

6470	11CB 14
6471	11CB 16
6472	11CB 17
6473	11CB 18

Study Handler AdRKB 3.75x10 Time 6wks

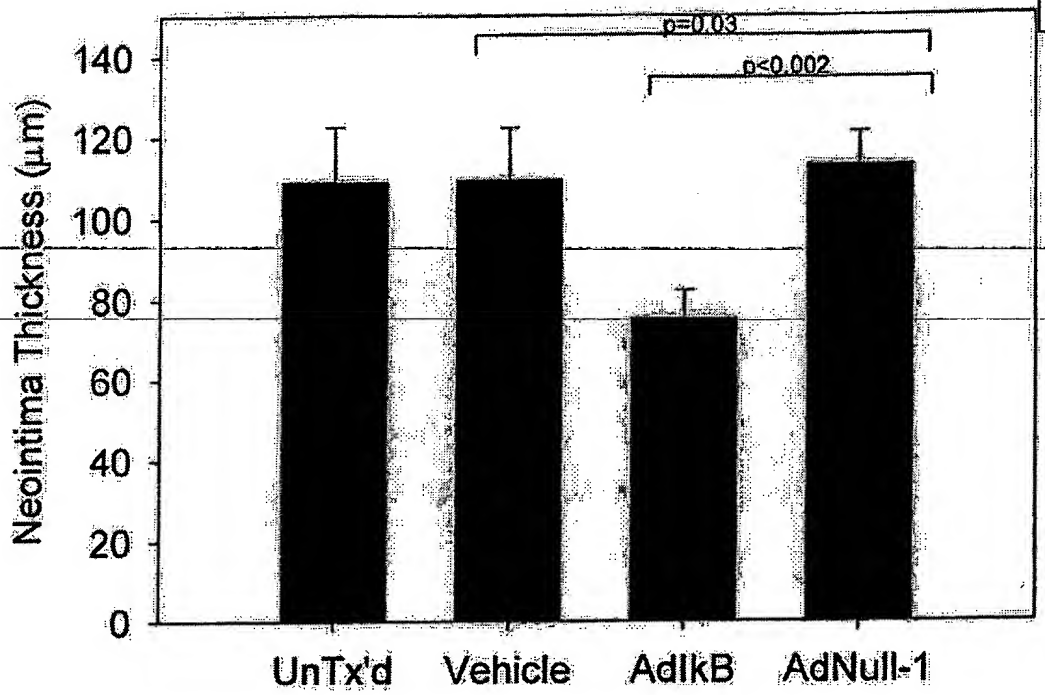
6700	11CB 14
6471	11CB 16
6472	11CB 17
6473	11CB 18

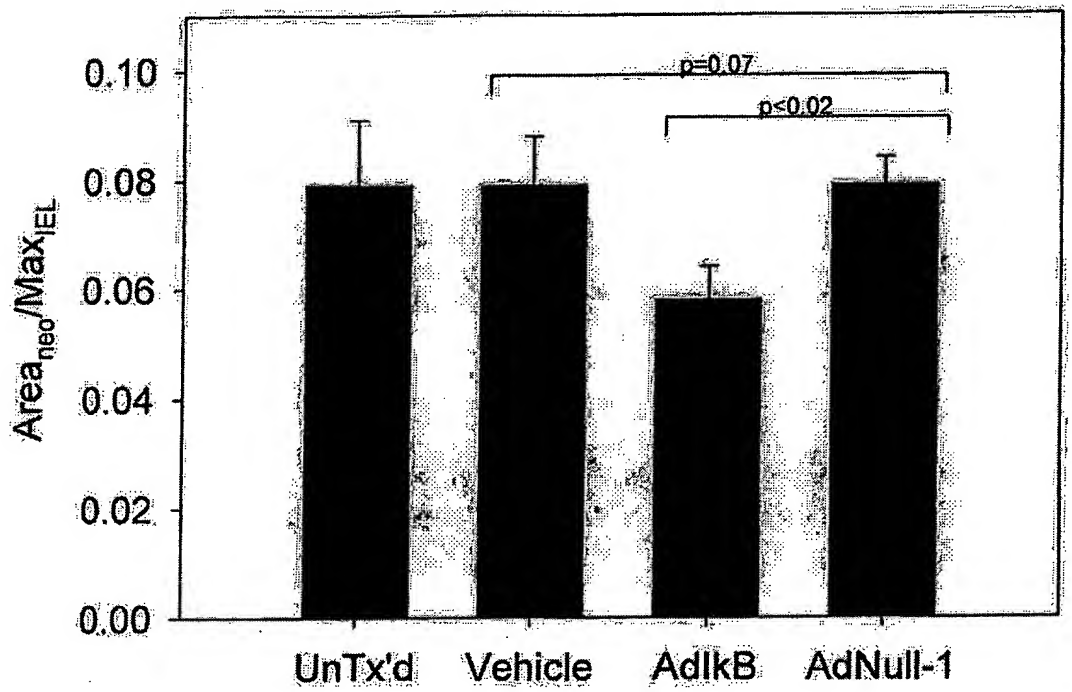
Sent APIO

To Page No.

Witnessed & Understood by me, Date Invented by Date Recorded by

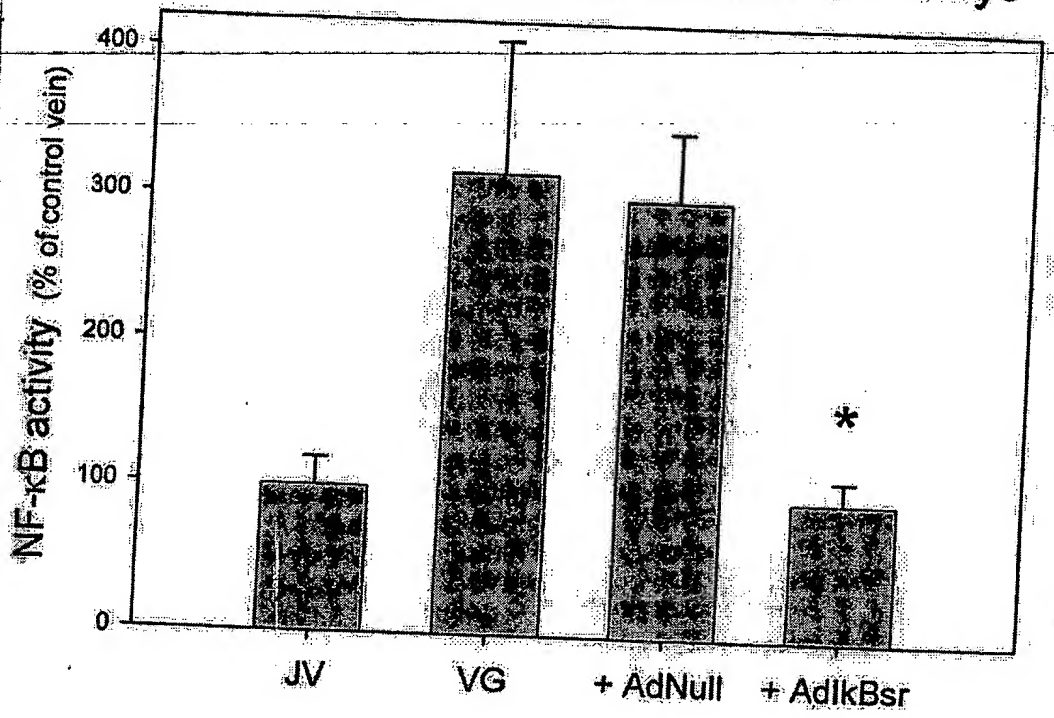
Figure 19


p=0.03
p<0.002
Neointima Thickness (µm)
140
120
100
80
60
40
20
0
UnTx'd
Vehicle
AdIkB
AdNull-1


p=0.07
p<0.02
Area$_{neo}$/Max$_{IEL}$
0.10
0.08
0.06
0.04
0.02
0.00
UnTx'd
Vehicle
AdIkB
AdNull-1

Project No.
Book No. TITLE

Figure 20



* P=0.014, as compared to AdNull-transduced vein graft

Reattempt @ Transducing HUVEC ≥ 7 days. Kits:

1) Jet PEI - RGD (Qbiogene Molecular Biology)

2) Tfx - 50 (Promega)

Witnessed & Understood by me, | Date | Invented by: | Recorded by